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Enhanced natural killer cell activity is found in exposed uninfected recipients of hepatitis C-contaminated blood

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Title: Enhanced natural killer cell activity is found in exposed uninfected recipients of hepatitis C-contaminated blood

Short title: NK cells in EU blood recipients

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Abstract

Background

A minority of injecting drug users, termed exposed uninfected (EU), are resistant to hepatitis C (HCV) infection despite repeated low-dose exposures. We identify for the first time a cohort of blood recipients who remained uninfected despite large-dose exposure to HCV-contaminated blood and characterise immune factors that may confer protection.

Methods

Of 1340 blood recipients from the Look Back database who were transfused HCV-contaminated blood, we identified 8 who remained uninfected. In these 8 EUs, we characterised their natural killer (NK) cell populations and HCV-specific T cell responses. Findings were compared with 10 spontaneous resolvers of HCV infection, 10 patients with chronic HCV infection, and 10 healthy controls.

Results

EUs had significantly greater numbers of NK cells with the activating receptor NKp30+ on $CD56^{bright}$ and $CD56^{dim}$ subsets compared with other groups ($p<0.05$). Following interleukin-2 activation, NK cells of EUs had enhanced cytotoxicity that positively correlated with NKp30 expression ($p=0.02$). Differences in NKp80 and KIR2DL3 expression were also observed. HCV-specific T cell responses were observed in some EUs but of low amplitude.

Conclusion

Exposure without infection following transfusion of HCV-contaminated blood is a very rare phenomenon and suggests a high level of resistance to infection. Enhanced NK cell activation and killing, with weak HCV-speecific T cell responses, were observed many years after

exposure in uninfected recipients and may contribute to protection from HCV acquisition, although additional protective factors are being sought in this important cohort.

Keywords

Hepatitis C; exposed uninfected; natural killer cells; innate immunity; natural cytotoxicity receptor; NKp30

Hepatitis C virus (HCV) is an important cause of liver disease with a propensity to establish chronic infection. Only a minority of individuals are able to spontaneously clear the virus, which relies on the host mounting a successful immune response [1-3]. Not all individuals exposed to HCV, however, develop infection. We and others have previously reported on a novel phenotype of injecting drug users (IDUs) who have no evidence of infection, such as viraemia or anti-HCV antibodies (Ab), despite repeated HCV exposure through high-risk practices [4-16]. We have termed these individuals exposed uninfected (EU) [17].

Thus far, studies on EUs have focused on IDUs exposed to low doses of the virus. The phenomenon of exposure without infection has not been previously described in individuals parenterally exposed to large doses of HCV, such as following transfusion of contaminated blood products. In this study, we have identified and characterised a novel cohort of recipients of HCV-contaminated blood products who have no clinical or serological evidence of infection and who appear to be immunologically distinct from spontaneous resolvers (SRs). Innate and adaptive immune factors that might contribute to this protection against HCV were examined, with a focus on natural killer (NK) cells.

NK cells are defined as CD3- CD56+ and play a key part in the early innate response to viral infections. Unlike T and B cells, they do not require priming and so are able to rapidly kill virus-infected cells [18]. Their activation status is governed by surface receptors with activating and inhibitory functions [19]. EU IDUs appear to be immunologically distinct from those with resolved or chronic infection, displaying an enhanced innate immune response [17,20] attributed to increased NK cell activity. Two studies followed a population of IDUs at high risk for HCV exposure and observed that in IDUs who remained uninfected, their NK cells demonstrated increased cytotoxic activity and interferon-gamma (IFN-γ) production

when compared with IDUs who became infected [7,8]. EU IDUs also possessed NK cells with a specific receptor profile, which might represent a subset with a favourable phenotype that are more readily activated to kill, thereby preventing establishment of infection [7-9]. These findings have thus far been demonstrated in EUs exposed to only low doses of HCV. This study reports on the immune characteristics of a novel cohort of EUs exposed to a much larger inoculum of virus via blood transfusion.

Materials and methods

Study population

The study population consisted of blood recipients identified from the NHS Look Back programme as being exposed to HCV via blood transfusion pre-1991, which was prior to universal HCV donor screening in the UK.

The Look Back programme was established in 1995 to trace and test recipients of blood products previously donated by blood donors who were subsequently found to be HCV positive when screening was introduced. We have reviewed the Look Back cohort to identify those who did not develop any evidence of HCV infection despite confirmed high-dose exposure to HCV-contaminated blood products. We compared findings in these exposed uninfected cases with others who developed chronic infection or spontaneously resolved infection.

EU cohort

From a total of 1340 blood recipients identified in the Look Back programme, eight EU cases were stringently selected. The following criteria were used:

1) Definitive history of exposure to HCV from blood products;

2) Both HCV Ab and HCV ribonucleic acid (RNA) undetectable;

3) All EU cases received blood from HCV-infected donors whose blood donations had transmitted HCV to at least one recipient before and one recipient after donating blood to that EU individual, providing confirmation that the donor was infectious at the time of donation; or, the EU individual had to have received part of a blood donation that was responsible for infecting another recipient, thus confirming the donation itself was contaminated.

SR cohort

The SR cohort consisted of 10 blood recipients recruited from the Look Back programme who spontaneously cleared HCV following infection via blood transfusion, defined as anti-HCV Ab positive and HCV RNA negative.

Chronic infection cohort

A cohort of 10 treatment naïve patients with blood transfusion-acquired genotype 1 chronic HCV infection (anti-HCV Ab and HCV RNA positive for at least six months) were recruited from the South West Liver Unit clinic at Derriford Hospital, Plymouth.

Healthy control cohort

A cohort of 10 healthy controls with no risk factors for HCV was also recruited from Derriford Hospital and University of Plymouth.

Timing of HCV testing

All EU and SR blood recipients recruited from the Look Back programme were tested for HCV at the time of the programme (1995-1997) for anti-HCV Ab by a third-generation enzyme immunoassay (EIA) and HCV RNA by polymerase chain reaction (PCR). Median time from transfusion to the initial anti-HCV Ab testing was 7.4 years (interquartile range [IQR] 6.1-9.3) in EUs and 7.1 years (IQR 4.9-10.7) in SRs.

At study entry, each blood recipient was re-tested for anti-HCV Ab using a fourth-generation EIA assay, Monolisa HCV Ag-Ab Ultra (Bio-Rad Laboratories, Hertfordshire, UK), and for HCV RNA by PCR using the Cobas Ampliprep/Cobas Taqman HCV Test v2.0 (Roche, California, United States; lower limit of detection 15 IU mL-1).

Ethics

Ethics approval was granted by the UK Research Ethics Committee. All procedures were performed in accordance with the ethical standards of the Helsinki Declaration (1964, amended 2008) created by the World Medical Association. All participants provided informed written consent.

Peripheral blood mononuclear cells (PBMCs) isolation

PBMCs were isolated from whole blood using Ficoll (Sigma-Aldrich, Dorset, UK) density gradient centrifugation and cryopreserved for subsequent analyses [4,5].

Flow cytometry

Flow cytometry was performed using a four-channel BD Accuri C6 instrument (New Jersey, USA). Thawed PBMCs ($1x10^6$) were stained with the appropriate fluorescent-labelled monoclonal Ab/isotype, washed, and analysed. Ab labelled with FITC/PE/PerCP-Cy5.5/APC specific for the following human cell surface antigens were used: CD3, CD56, and CD16 from BD Biosciences (Oxford, UK); and KIR2DL1, CD158b, KIR2DL3, KIR2DL4, KIR2DS4, KIR3DL1, KIR3DL2, NKp30, NKp44, NKp46, NKG2A, NKG2C, and NKG2D from R&D Systems (Oxford, UK).

Cytotoxicity assay

Natural and interleukin (IL) 2-induced cytotoxicity of NK cells were measured against the target cell K562. NK cells were isolated from PBMCs using negative selection with the NK Cell Isolation Kit (Miltenyi Biotec, Surrey, UK). Isolated NK cells were cultured with/without IL2 (25 ng mL-1; R&D Systems) for 48 hours. Following culture, K562 cells, labelled with carboxyfluorescein succinimidyl ester (Fisher-Scientific, Leicester, UK), were

added to NK cells at an effector-to-target cell ratio of 0:1 (negative control) or 10:1 and incubated for four hours. Finally, 7-aminoactinomycin D (BD Biosciences) was added prior to flow cytometry.

IFN-γ production

Thawed PBMCs were stimulated with phorbol 12-myristate 13-acetate (Sigma-Aldrich) and ionomycin (Sigma-Aldrich) in the presence of brefeldin A (BD Golgi Plug, BD Biosciences) prior to incubation for four hours. After stimulation, cells were stained for CD3 and CD56 and then fixed and permeabilised using the BD Cytofix/Cytoperm Plus Fixation/Permeabilisation Kit (BD Biosciences). Following this, the cells were stained with fluorescent-labelled Ab for intracellular IFN-γ (BD Biosciences), washed, and analysed on the flow cytometer. Unstimulated PBMCs served as the negative control.

IFN-γ enzyme-linked immunospot (ELISpot) assay

ELISpot assays were performed as previously described [4,5]. Briefly, 200,000 PBMCs per well were cultured in triplicate with HCV peptides. The peptides spanned the entire genome of HCV genotypes 1b and 3a (BEI Resources, Virginia, United States) and were grouped into eight pools per genotype (core, E1, E2, p7 and NS2, NS3, NS4, NS5a, NS5b). Positive controls were: leucoagglutinin (PHA-L; Sigma-Aldrich); cytomegalovirus, Epstein-Barr virus, and influenza virus peptide pool (ProImmune, Oxford, UK); and cytomegalovirus pp65 peptide pool (ProImmune). Cells cultured in media alone acted as the negative control. After 16 hours of culture, the PBMCs were plated onto an ELISpot plate (Merck Millipore, Hertfordshire, UK) coated with IFN-γ Ab (BD Biosciences).

The mean number of spots was calculated for each triplicate. The number of spots in the negative control wells was subtracted from that of the antigen-treated wells and results expressed as spot-forming units per million cells (SFU per 10^6 PBMCs). Assays with high background (negative control greater than five spots per well) or absent PHA-L responses were excluded. A response was defined as positive if the mean response in the antigen-treated well was more than twice the background value in the negative well and greater than three standard deviations above the mean response in the corresponding well of the healthy controls.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics v20. Results were expressed as medians unless stated otherwise. Categorical data were compared using Fisher's exact test. Continuous data between two cohorts were compared using Mann-Whitney-U test. With more than two cohorts, continuous data were compared using Kruskal-Wallis test. A statistically significant Kruskal-Wallis test was followed by post-hoc analysis using Dunn's test for multiple comparisons between cohorts. Adjusted *p* values were calculated to correct for multiple comparisons. Pearson product-moment correlation coefficient (*r*) was calculated for correlation analysis. A line of best fit was drawn using linear regression. Statistical significance levels were determined by two-tailed tests ($p < 0.05$).

Results

Of the 1340 blood recipients identified as having received a contaminated blood product, 13 (0.97%) met the inclusion criteria for EU. Eleven were alive, of which eight were recruited into the study.

Baseline demographics and transfusion details of the eight EUs are shown in Table 1. Median age at time of transfusion was 21 years (IQR 13-32). There was no history of post-transfusion hepatitis. One participant, EU5, had biopsy-proven non-alcoholic fatty liver disease; the remaining seven had no history of liver disease or abnormal liver enzymes. There was a range of active medical conditions. No participant was on steroids or immunosuppression at the time of transfusion or recruitment.

Table 2 shows the donor-recipient records for each of the donors that donated blood to our EUs. In five cases, the donors (X1, X2, X3, X5, X7) infected other recipients both before and after donating blood to our EUs (EU1, EU2, EU3, EU5, EU7, respectively), confirming the donors were infectious during this time. In the remaining three cases, donors X4, X6, and X8 each made a donation that was separated into components and transfused to more than one recipient (X4 to EU4 and R9; X6 to EU6 and R13; X8 to EU8 and R19). The other recipients developed infection, thereby confirming the donation itself, which the three EUs had also received, was contaminated. In all cases, the genotypes of the infected recipients matched those of their respective donors.

A further 10 participants were recruited into each of the remaining three cohorts. There were no significant differences in the baseline characteristics between the four cohorts. Median ages at study entry were: 45 years (IQR 36-59) for the EU cohort, 67 years (IQR 47-71) for

the SR cohort, 52 years (IQR 43-61) for the cohort with chronic infection, and 46 years (IQR 39-53) for the healthy controls. In the EU, chronic infection, and healthy control cohorts, 50% were male; in the SR cohort, 40% were male.

EU blood recipients possessed higher numbers of NKp30+ NK cells

Fourteen activating and inhibitory NK cell receptors were studied on flow cytometry. NK cells were visualised as CD3- CD56+. The two functional subsets of NK cells, $CD56^{bright}$ and $CD56^{dim}$, were defined by the varying intensities of CD56 expression. EUs were observed to have a higher proportion of NK cells than healthy controls and those with chronic infection (Figure 1); however, there was no difference in the distribution of NK cell subsets between EUs and the other cohorts, suggesting that the higher relative frequencies of NK cells in EUs were due to an increase in both $CD56^{bright}$ and $CD56^{dim}$ cells.

The numbers of NKp30+ NK cells were significantly higher in EUs than in the other cohorts – vs. SRs (p=0.002), vs. healthy controls (p=0.01), and vs. patients with chronic infection (p=0.04) (Figure 2A). This increased expression of NKp30 in EUs was observed across both subsets. EUs had significantly greater numbers of $CD56^{dim}$ NKp30+ cells when compared to SRs (p=0.007) and healthy controls (p=0.03), and trending towards significance when compared with the chronic infection cohort (p=0.06) (Figure 2B). In the $CD56^{bright}$ subset, numbers of NKp30+ cells were highest in the EUs, significant against SRs (p=0.047), healthy controls (p<0.001), and those with chronic infection (p=0.02) (Figure 2C).

Other differences in receptor expression were also observed. There were significantly higher levels of NKp80+ NK cells in EUs and SRs than in healthy controls, although not so when compared with the chronic infection cohort (Figure 3A). The expression of the inhibitory NK

cell receptors was also examined. The only significant difference was observed with the killer cell immunoglobulin-like receptor, KIR2DL3, which was preferentially expressed on $CD56^{bright}$ NK cells of both EUs and SRs (Figure 3B).

NKp30-^{high} NK cells demonstrated enhanced IL2-induced cytotoxicity

Cytotoxic activity of isolated NK cells (>90% purity) was determined to confirm whether differences in receptor expression were associated with enhanced killing potential. NK cells at rest had little cytotoxic activity and no differences were detected between cohorts. Following IL2 activation, NK cells of EUs had greater cytotoxicity than the other cohorts – vs. SRs (p=0.06), vs. healthy controls (p=0.01), and vs. those with chronic infection (p=0.04) (Figure 4A). As NKp30 was the only receptor that was preferentially upregulated in EUs, we looked for a potential relationship between NKp30 expression and NK cell cytotoxicity. IL2-induced NK cell cytotoxicity was positively correlated with NKp30 expression, although this correlation was weak ($r=0.38$; p=0.02) (Figure 4B).

NK cells can kill virus-infected cells indirectly by rapidly producing IFN-γ. At rest, very few NK cells produced IFN-γ with no differences observed between cohorts. Following stimulation, NK cell production of IFN-γ was increased in all subjects, although to a lesser extent in those with chronic infection. IFN-γ production by $CD56^{dim}$ and $CD56^{bright}$ NK cells in EUs was no different from SRs and healthy controls.

T cell-mediated IFN-γ responses to HCV

T cell responses to genotypes 1b and 3a were assessed in the eight EUs and compared with eight healthy controls. Positive IFN-γ responses were observed only in the EU group, with 4/8 demonstrating a positive response to at least one HCV-related peptide compared with

none in the healthy control group. Although the positive responses in the four EUs were weak, they were all multi-specific i.e. directed against multiple HCV proteins, which support true exposure rather than cross-reactivity with a non-HCV epitope. HCV proteins that were targeted were genotype 1b core, NS3, NS4, and NS5B proteins, and genotype 3a core, NS3, and NS5A proteins. The median times from HCV exposure to ELISpot testing in EUs were not significantly different between those with positive responses (23.6 years) and those without (25.3 years).

Discussion

This study describes a novel cohort of blood recipients with no clinical or serological evidence of infection despite verified exposure to HCV-contaminated blood. This is the first report of exposure without infection in the setting of large-volume intravenous inoculation of HCV with 8 cases identified and traced after meticulous interrogation of the records from a well conducted national look back exercise dating back to the 1990's. Prior to this, exposure to HCV that did not result in infection had only been reported following much smaller inocula through IDU [4-16], sexual contact [21-23], or needle stick injury [24]. Transfusion-related HCV is, by and large, historical and so this cohort has considerable potential to advance our understanding of how high level resistance to HCV infection can be conferred.

We have shown that EU blood recipients have distinct differences in their NK cell phenotype and function. EUs had greater proportions of NK cells in the periphery, with a relative increase in both $CD56^{bright}$ and $CD56^{dim}$ subsets. One of the main findings was an activated receptor phenotype in EUs, with greater numbers of NKp30+ cells in both the $CD56^{bright}$ and $CD56^{dim}$ subsets, significantly higher than in the other groups. Thus, EU blood recipients not only possessed higher frequencies of NK cells, but their NK cells were richer in NKp30 compared to blood recipients with resolved or chronic infection. On functional analysis, activated NK cells in EUs displayed greater killing potential, with increased cytotoxicity but similar IFN-γ production. This enhanced killing potential of NK cells in EUs positively correlated with the expression of NKp30. These findings mirror those of two prospective studies conducted on IDUs; Sugden et al. [7] and Golden-Mason et al. [8] reported that increased NK cell cytotoxicity protected IDUs exposed to HCV from becoming infected and this enhanced cytotoxicity was associated with higher expression of NKp30. This study, therefore, supports NKp30-mediated NK cell cytotoxicity as a potential mechanism by which

individuals exposed to HCV are protected from infection, and appears to be relevant in both low- and high-dose exposures.

Other less striking differences were observed. NKp80 has an activating function, possibly by acting as co-receptor with other activating receptors [25]. EUs and SRs had significantly higher frequencies of NKp80+ NK cells than healthy controls, although levels were not statistically different when compared with those chronically infected. The significance of increased NKp80 levels is unclear; NKp80 has not previously been implicated in the immune defence against HCV. The levels of NKp80 did not correlate with cytotoxic activity, which could support the hypothesis that NKp80 alone has a limited role in NK cell activation, but acts instead as a co-receptor to potentiate the effect of a key receptor, such as NKp30.

KIR2DL3 has been associated with a favourable outcome following infection. HCV-infected IDUs have a higher chance of spontaneous clearance if they are homozygous for KIR2DL3 and HLA-C1. KIR2DL3 is a weaker inhibitor of NK cell activation than other inhibitory KIRs; thus, NK cells carrying this receptor are more easily activated [26]. The beneficial effect of KIR2DL3 and HLA-C1 extends beyond resolving infection, with our group and others showing that it may also play a part in preventing infection in IDUs exposed to HCV [9,13,15]. We now find KIR2DL3 to be associated with a favourable outcome in the setting of transfusion-related HCV exposure. Increased expression of KIR2DL3 was detected in both EUs and SRs, but only in the $CD56^{bright}$ subset. KIRs are usually expressed at minimal levels on $CD56^{bright}$ NK cells. Hence, upregulation of KIR2DL3 might render $CD56^{bright}$ cells more easily activated and enhance their functional activity against HCV.

In this study, half of EU blood recipients demonstrated IFN-γ-producing T cell responses to HCV, similar to rates reported in both our [5] and other [7,10-12,24] IDU EU cohorts, where up to 60% have demonstrable T cell responses. Whilst these responses are often to multiple viral antigens, they are typically much weaker than those seen in SRs and the degree to which T cell responses confer protection from HCV infection is controversial. A nested case control series from a prospectively studied cohort of IDUs in Australia revealed no differences in T cell responses between those who developed incident HCV infection and those who remained uninfected, suggesting that whilst HCV-specific cellular immunity is prevalent in EUs, it does not correlate with protection [7]. Thus, whilst the presence of low-level HCV-specific T cell responses in half of our EU blood recipients provides further confirmation of HCV exposure, any role they have in protection remains unclear. The absence of response in the remaining EU blood recipients is consistent with previous studies that showed T cell responses waning over time [4,6], with HCV exposure in these recipients dating back over 20 years.

There are some limitations to this study. We defined EUs as being HCV antibody and HCV RNA negative. However, as the first testing for HCV antibodies took place some years after exposure, it is possible that they were infected but lost HCV antibodies over time. This has been reported in SRs, but typically 18-20 years after a low dose inoculation from anti-D immunoglobulin [27], whereas EUs identified in this cohort were all tested within 10 years from exposure. The sample size was limited by the small number of uninfected individuals that met the very strict definition of HCV exposure. EU blood recipients represent an extremely rare cohort and therefore, any study examining this unique group will be limited by small numbers and the difficulties that this poses. Furthermore, our results, like other studies on EU cohorts [4-16], were based on experiments using peripheral blood samples to obtain

NK and T cells, when we know that the immune response to the virus is reliant on intrahepatic immune cells. However, to study intrahepatic immune cells would require invasive sampling of the liver, which is difficult to justify in EUs who are otherwise healthy.

In summary, we have defined a novel cohort with a uniquely high level of resistance to HCV infection. This small group of cases, identified and traced from a painstaking review of a much larger cohort of HCV-exposed blood recipients, remained uninfected despite a clear history of high-dose parenteral exposure to HCV-contaminated blood. These EU blood recipients, similar to IDU EU cases, demonstrate distinct differences in their innate immunity, with an activated NK cell phenotype and enhanced cytotoxicity, together with low level adaptive HCV-specific T cell responses in half of these cases identified many years after the HCV exposure. It remains unclear if these differences alone are sufficient to explain the high level resistance demonstrated by these unusual cases and further study of this important group of cases to identify other potential mechanisms of protection from HCV infection are ongoing.

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Statement of interests

Authors declaration of personal interests:

None reported for all authors.

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References

1. Seeff LB. Natural history of chronic hepatitis C. Hepatology **2002**; 36: S35-46.
2. Rehermann B. Hepatitis C virus versus innate and adaptive immune responses: a tale of coevolution and coexistence. J Clin Invest **2009**; 119: 1745-54.
3. Thimme R, Binder M, Bartenschlager R. Failure of innate and adaptive immune responses in controlling hepatitis C virus infection. FEMS Microbiol Rev **2012**; 36: 663-83.
4. Thurairajah PH, Hegazy D, Demaine A, Kaminski ER, Cramp ME. Loss of virus-specific T-cell responses in HCV exposed uninfected injection drug users with drug rehabilitation. J Infect Dis **2011**; 203: 847-53.
5. Thurairajah PH, Hegazy D, Chokshi S, et al. Hepatitis C virus (HCV)–specific T cell responses in injection drug users with apparent resistance to HCV Infection. J Infect Dis **2008**; 198: 1749-55.
6. Cameron B, Galbraith S, Li H, Lloyd A. Correlates and characteristics of hepatitis C virus-specific T-cell immunity in exposed uninfected high-risk prison inmates. J Viral Hepat **2013**; 20: e96-106.
7. Sugden PB, Cameron B, Mina M, Lloyd AR. Protection against hepatitis C infection via NK cells in highly-exposed uninfected injecting drug users. J Hepatol **2014**; 61: 738-45.
8. Golden-Mason L, Cox AL, Randall JA, Cheng L, Rosen HR. Increased natural killer cell cytotoxicity and NKp30 expression protects against hepatitis C virus infection in high-risk individuals and inhibits replication in vitro. Hepatology **2010**; 52: 1581-9.
9. Thoens C, Berger C, Trippler M, et al. KIR2DL3(+)NKG2A(-) natural killer cells are associated with protection from productive hepatitis C virus infection in people who inject drugs. J Hepatol **2014**; 61: 475-81.

10. Freeman AJ, Ffrench RA, Post JJ, et al. Prevalence of production of virus-specific interferon-gamma among seronegative hepatitis C-resistant subjects reporting injection drug use. J Infect Dis **2004**; 190: 1093-7.
11. Mizukoshi E, Eisenbach C, Edlin BR, et al. Hepatitis C virus (HCV)–specific immune responses of long-term injection drug users frequently exposed to HCV. J Infect Dis **2008**; 198: 203-212.
12. Zeremski M, Shu MA, Brown Q, et al. Hepatitis C virus-specific T-cell immune responses in seronegative injection drug users. J Viral Hepat **2009**; 16: 10-20.
13. Zuniga J, Romero V, Azocar J, et al. Protective KIR–HLA interactions for HCV infection in intravenous drug users. Mol Immunol **2009**; 46: 2723-27.
14. Pelletier S, Drouin C, Bédard N, Khakoo SI, Bruneau J, Shoukry NH. Increased degranulation of natural killer cells during acute HCV correlates with the magnitude of virus-specific T cell responses. J Hepatol **2010**; 53: 805-16.
15. Knapp S, Warshow U, Hegazy D, et al. Consistent beneficial effects of killer cell immunoglobulin-like receptor 2DL3 and group 1 human leukocyte antigen-C following exposure to hepatitis C virus. Hepatology **2010**; 51: 1168-75.
16. Knapp S, Warshow U, Ho KMA, et al. A polymorphism in IL28B distinguishes exposed, uninfected individuals from spontaneous resolvers of HCV infection. Gastroenterology **2011**; 141: 320-5.
17. Shawa IT, Felmlee DJ, Hegazy D, Sheridan DA, Cramp ME. Exploration of potential mechanisms of hepatitis C virus resistance in exposed uninfected intravenous drug users. . J Viral Hepat. **2017** May 5. doi: 10.1111/jvh.12720. [Epub ahead of print]
18. Cheent K, Khakoo SI. Natural killer cells and hepatitis C: action and reaction. Gut **2011**; 60: 268-78.

19. Mondelli MU, Varchetta S, Oliviero B. Natural killer cells in viral hepatitis: facts and controversies. Eur J Clin Invest **2010**; 40: 851-63.
20. Warshow UM, Riva A, Hegazy D, et al. Cytokine profiles in high risk injection drug users suggests innate as opposed to adaptive immunity in apparent resistance to hepatitis C virus infection. J Viral Hepat 2012; 19: 501-8.
21. Bronowicki J, Vetter D, Uhl G, et al. Lymphocyte reactivity to hepatitis C virus (HCV) antigens shows evidence for exposure to HCV in HCV-seronegative spouses of HCV-infected patients. J Infect Dis **1997**; 176: 518-22.
22. Kamal SM, Amin A, Madwar M, et al. Cellular immune responses in seronegative sexual contacts of acute hepatitis C patients. J Virol **2004**; 78: 12252-8.
23. Rivière Y, Montange T, Janvier G, et al. Hepatitis C virus-specific cellular immune responses in individuals with no evidence of infection. Virol J **2012**; 9: 76.
24. Koziel MJ, Wong DKH, Dudley D, Houghton M, Walker BD. Hepatitis C virus-specific cytolytic T lymphocyte and T helper cell responses in seronegative persons. J Infect Dis **1997**; 176: 859-66.
25. Vitale M, Falco M, Castriconi R, et al. Identification of NKp80, a novel triggering molecule expressed by human NK cells. Eur J Immunol **2001**; 31: 233-42.
26. Khakoo SI, Thio CL, Martin MP, et al. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. Science **2004**; 305: 872-4.
27. Takaki A, Wiese M, Maertens G, et al. Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. Nat Med **2000**; 6: 578–82.

Table 1. Baseline demographics and clinical characteristics of the eight EU participants

ID	Age at study entry (yrs)	Sex M/F	Ethnicity	Date transfused	Type of product	Indication	Other HCV risks	Symptoms of hepatitis	Abnormal liver enzymes	Active medical conditions	Immuno suppression
EU1	52	M	White British	05/08/1982	RBC	Trauma	No	No	No	Hypertension	No
EU2	41	F	White British	07/01/1985	Platelet	Childhood ITP	No	No	No	No	No
EU3	32	M	White British	07/04/1987	Whole blood	Facial surgery	No	No	No	Gout	No
EU4	72	F	White British	01/02/1989	RBC	Hysterectomy	No	No	No	Hypertension, type II diabetes	No
EU5	61	F	White British	20/02/1989	Whole blood	Menorrhagia	No	No	NAFLD	Hypothyrodism on replacement	No
EU6	48	M	Afro-Caribbean British	21/06/1990	RBC	Renal failure	No	No	No	Haemodialysis	No
EU7	42	F	Afro-Caribbean British	29/10/1990	RBC	Sickle cell anaemia	No	No	No	Sickle cell anaemia	No

EU8	34	M	White British	25/11/1990	RBC	Bone surgery	No	No	No	Asthma	No

EU, exposed uninfected; yrs, years; M, male; F, female; HCV, hepatitis C; RBCs, red blood cells; ITP, idiopathic thrombocytopaenic purpura; NAFLD, non-alcoholic fatty liver disease

Table 2. Donations made by the donors of the eight EU participants and their corresponding recipients

Donor	Donation	Recipient	Transfusion date	HCV test result by Lookback Programme
X1	1	R1	17/03/1981	Infected – chronic HCV
	2	**EU1**	**05/08/1982**	**Uninfected**
	3	R2	05/01/1983	Infected – chronic HCV
X2	1	R3	03/03/1984	Infected – chronic HCV
	2	**EU2**	**07/01/1985**	**Uninfected**
	3	R4	29/04/1986	Infected – chronic HCV
	4	R5	15/02/1987	Infected – spontaneous clearance
X3	1	R6	16/09/1983	Infected – spontaneous clearance
	2	R7	22/09/1986	Infected – chronic HCV
	3	**EU3**	**07/04/1987**	**Uninfected**
	4	R8	16/10/1987	Infected – spontaneous clearance
X4	1	R9*	17/01/1989	Infected – chronic HCV
	1	**EU4***	**01/02/1989**	**Uninfected**
X5	1	R10	03/12/1987	Infected – spontaneous clearance
	2	R11	08/09/1988	Infected – chronic HCV
	3	**EU5**	**20/02/1989**	**Uninfected**
	4	R12	26/01/1990	Infected – chronic HCV
X6	**1**	**EU6***	**21/06/1990**	**Uninfected**
	1	R13*	24/06/1990	Infected – chronic HCV
X7	1	R14	12/12/1985	Infected – chronic HCV
	2	R15	03/08/1988	Infected – chronic HCV
	3	R16	17/01/1990	Infected – chronic HCV
	4	**EU7**	**29/10/1990**	**Uninfected**
	5	R17	28/03/1991	Infected – chronic HCV
X8	1	R18	11/04/1989	Infected – spontaneous clearance
	2	R19*	22/11/1990	Infected – spontaneous clearance

2	**EU8***	**25/11/1990**	**Uninfected**	

*Received blood product from the same donation

EU, exposed uninfected; HCV, hepatitis C

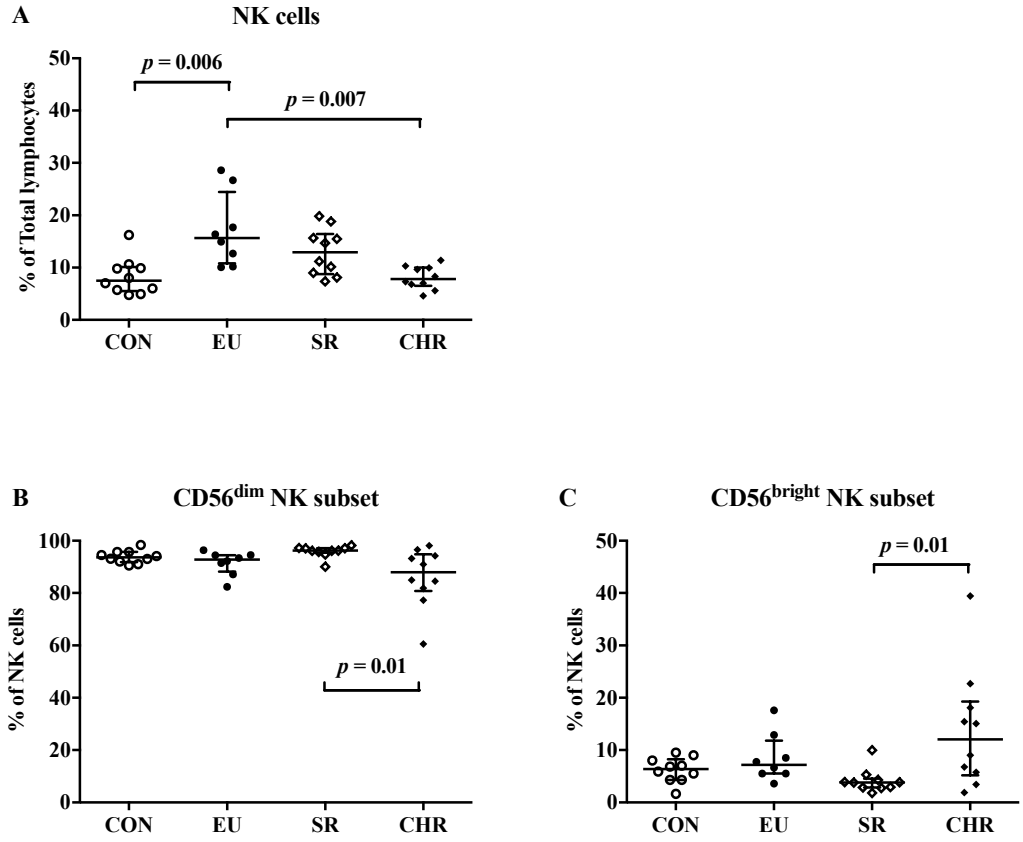
A
NK cells
% of Total lymphocytes
p = 0.006
p = 0.007
CON
EU
SR
CHR
B
CD56dim NK subset
% of NK cells
p = 0.01
C
CD56bright NK subset
% of NK cells
p = 0.01

Figure 1

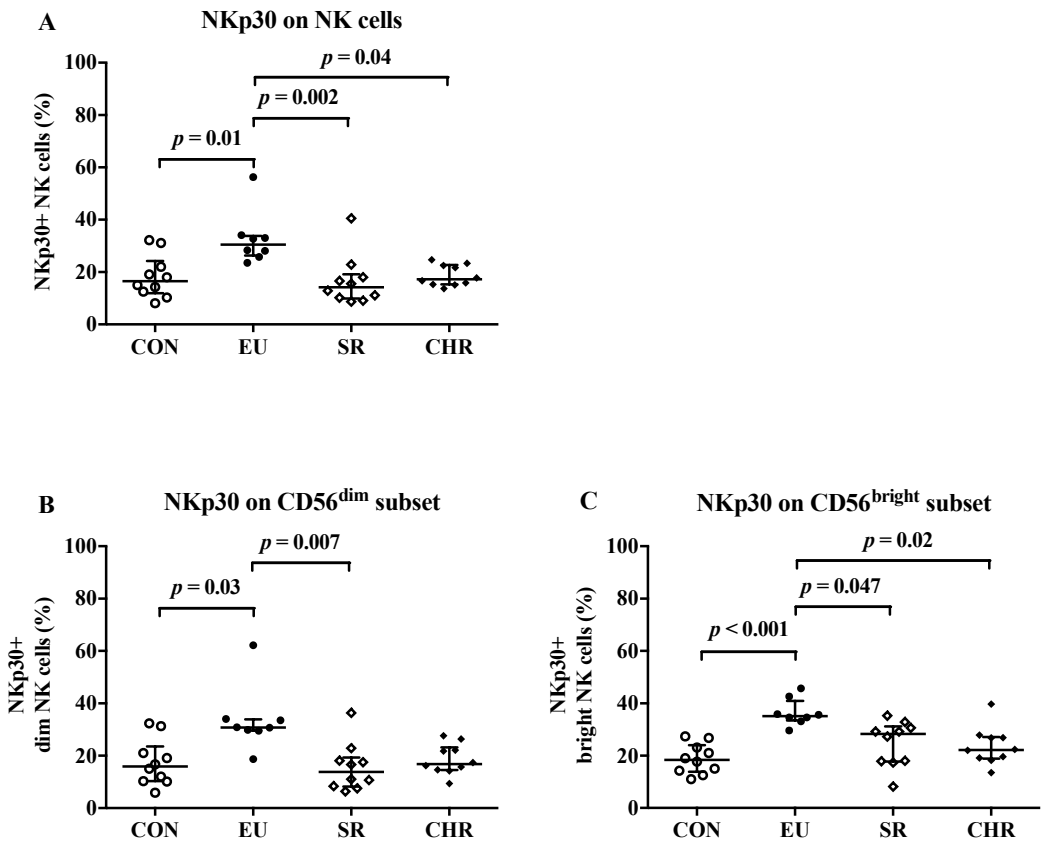
A
NKp30 on NK cells
NKp30+ NK cells (%)
p = 0.04
p = 0.002
p = 0.01
CON
EU
SR
CHR
B
NKp30 on CD56dim subset
NKp30+ dim NK cells (%)
p = 0.007
p = 0.03
C
NKp30 on CD56bright subset
NKp30+ bright NK cells (%)
p = 0.02
p = 0.047
p < 0.001

Figure 2

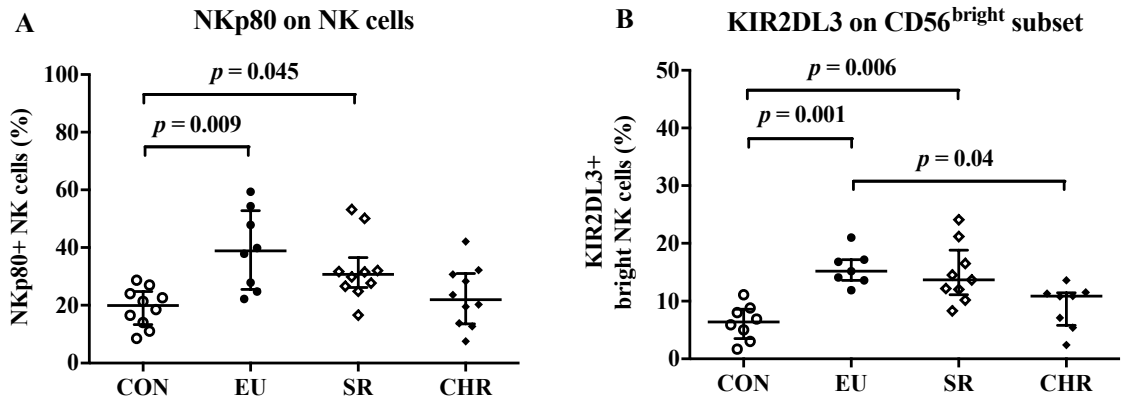
A
NKp80 on NK cells
NKp80+ NK cells (%)
p = 0.045
p = 0.009
100
80
60
40
20
0
CON
EU
SR
CHR
B
KIR2DL3 on CD56bright subset
KIR2DL3+ bright NK cells (%)
p = 0.006
p = 0.001
p = 0.04
50
40
30
20
10
0
CON
EU
SR
CHR

Figure 3

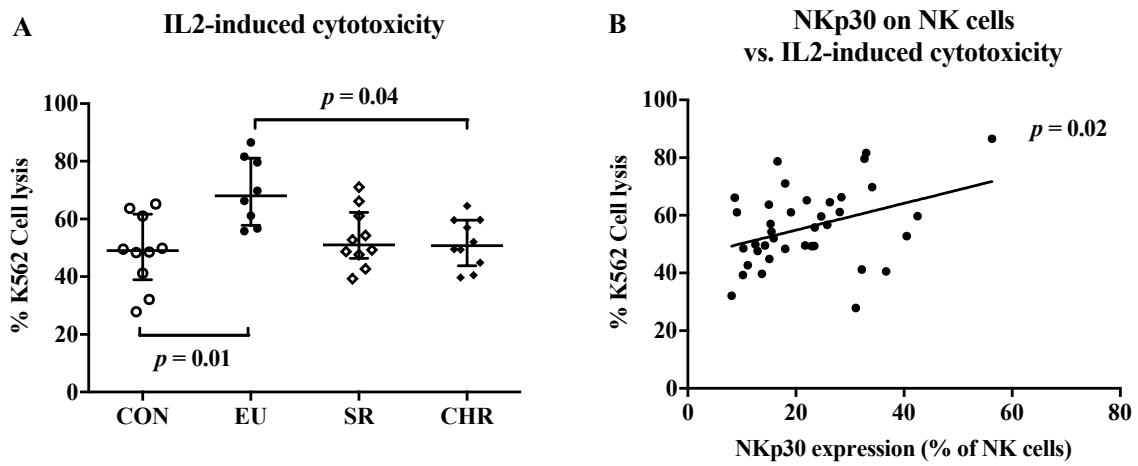
A
IL2-induced cytotoxicity
% K562 Cell lysis
100
80
60
40
20
0
p = 0.04
p = 0.01
CON
EU
SR
CHR
B
NKp30 on NK cells
vs. IL2-induced cytotoxicity
% K562 Cell lysis
p = 0.02
0
20
40
60
80
NKp30 expression (% of NK cells)

Figure 4